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Effect of Oxygen Free Radicals on Mg^{2+} Efflux from Erythrocytes

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Dedicated to Prof. Dr. Dr. H. Greiling on occasion of his 65th birthday

Summary: Hydrogen peroxide destroyed the Na^+/Mg^{2+} antiport in Mg^{2+}-loaded human and rat erythrocytes and increased the leakage of intracellular Mg^{2+} and K^+. These effects are opposite to the increase of Na^+/Mg^{2+} antiport and unchanged Na^+-independent Mg^{2+} efflux from erythrocytes of patients with cystic fibrosis score 3. Thus, the increase of Na^+/Mg^{2+} antiport in these patients is not caused by increased formation of free radicals.

Introduction

Mg^{2+} efflux from erythrocytes can be detected after loading the cells with Mg^{2+}. Mg^{2+} efflux from Mg^{2+}-loaded erythrocytes occurs by amiloride-sensitive electroneutral Na^+/Mg^{2+} antiport, and by Na^+-independent Mg^{2+} efflux which is accompanied by efflux of Cl^- for charge compensation. The latter is inhibited by high extracellular Cl^- ($[Cl^-]_o$) and by 4-acetamino-4'-isothiocyanatostilbene-2,2'-disulphonate (SITS) (1–3).

Both Mg^{2+} efflux systems are differently expressed in erythrocytes of various species. Na^+/Mg^{2+} antiport is highly expressed in rat erythrocytes, whereas Cl^--accompanied Mg^{2+} efflux is relatively more expressed in human than rat erythrocytes (4).

In preceding experiments we found increased Na^+/Mg^{2+} antiport in erythrocytes from patients with cystic fibrosis (5). Na^+/Mg^{2+} antiport correlated with the clinical score of the disease, being highest in erythrocytes from patients with score 3 but being not significantly increased in scores 1 and 2. Na^+-independent Mg^{2+} efflux, i. e. Mg^{2+} efflux in choline chloride or sucrose medium, was not affected (5). The increase in Na^+/Mg^{2+} antiport showed no obvious correlation to the DF 508 mutation (6) which is responsible for 70–80% of the genetic defect (7, 8).

In an investigation of Na^+/Mg^{2+} antiport in individual patients, this process was also found to be increased in a patient with thalassaemia (unpublished). Cystic fibrosis patients with score 3 were suffering from pulmonal infections. During infection, granulocytes show oxygen burst which is combined with the formation of H_2O_2 and oxygen free radicals. Oxygen free radicals produced by stimulated neutrophils can attack erythrocytes (9). Indeed, in cystic fibrosis patients the formation of free radicals was increased (10) and the free radical trapping capacity in plasma of cystic fibrosis patients was reduced (11).

Also, free α- or β-chains of haemoglobin, which are formed in thalassaemia, induce a higher rate of oxygen free radicals than haemoglobin (Hb) (12). Moreover, in amnion cells, oxygen radicals reduced $[Mg^{2+}]_i$, independent of $[Na^+]_o$ (13).

Therefore, we investigated the effect of H_2O_2 on Na^+-dependent and Na^+-independent Mg^{2+} efflux from Mg^{2+}-loaded normal rat and human erythrocytes. Part of the H_2O_2 can be converted into the very active and toxic $OH^\cdot$ via the Fe^{2+} catalysed *Fenton* reaction:

$$H_2O_2 + Fe^{2+} \rightarrow OH^\cdot + OH^- + Fe^{3+}.$$

Fe^{2+} may originate from Hb either directly or after liberation from Hb by H_2O_2 (14, 15). However, it is not known whether H_2O_2 or $OH^\cdot$ is the reactive oxygen species (9).

Materials and Methods

The experiments were performed with normal human erythrocytes (from one donor, J. V.) and rat erythrocytes from unstarved normal male Wistar rats weighing 200 g. Blood was taken (from rats under Nembutal anaesthesia (50 mg/kg i. p.) with a heparinized syringe, and centrifuged at 1000 *g* for 10 min. The plasma and buffy coat were aspirated and the red cells were washed twice with 150 mmol/l KCl.

The cells were loaded with Mg^{2+} by incubating a cell suspension (100 ml/l) for 30 min at 37 °C in KCl medium (in mmol/l: 140 KCl; 12 $MgCl_2$, 50 sucrose, 5 glucose, 30 Hepes-Tris, pH 7.4) with the addition of 6 µmol/l A23187 dissolved in dimethyl sulphoxide. For removal of the ionophore the cells were incubated four times in KCl medium plus 10 g/l bovine serum albumin for 10 min at 37 °C. Thereafter, the cells were suspended in KCl medium and 2 mmol/l Na azide was added to inhibit catalase. After 5 min, 1–20 µmol/l H_2O_2 was added as indicated and the cells were incubated at 37 °C for 45 min. Azide and H_2O_2 were removed by washing the cells twice in cold choline chloride medium (see below).

Mg^{2+} and K^+ efflux was measured by reincubating a cell suspension (100 ml/l) at 37 °C in Mg^{2+}-free medium. For reincubation, sucrose medium (in mmol/l: 350 sucrose, 5 glucose, 30 Hepes-Tris, pH 7.4), NaCl or choline chloride medium (substitution of KCl in KCl medium by 140 mmol/l NaCl or 140 mmol/l choline chloride) were used. At the beginning of reincubation and after 30 min, 0.5 ml aliquots of the cell suspensions were centrifuged for 1 min at 10 000 *g*. For Mg^{2+} determination, 100 µl supernatant was diluted with 1 ml 100 g/l trichloroacetic acid, 1.75 g/l $LaCl_3$, and Mg^{2+} was measured by atomic absorption spectrophotometry (Philips, SP9). K^+ was measured in the supernatant by flame photometry, after addition of appropriate amounts of LiCl (KLiNa-Flame, Beckman). Mg^{2+} efflux and K^+ efflux were calculated from the increase of Mg^{2+} and K^+ in the reincubation media. The efflux was related to the cell volume as determined by the haematocrit. As a control, the Mg^{2+} content of Mg^{2+}-loaded cells was measured. For measuring cellular Mg^{2+} content, the cells were washed twice with 150 mmol/l KCl, haemolysed by adding 750 µl H_2O, deproteinized by addition of 50 µl 750 g/l trichloroacetic acid, and centrifuged. Mg^{2+} was measured by atomic absorption spectrophotometry after dilution with 100 g/l trichloroacetic acid, 1.75 g/l $LaCl_3$.

Results

Treatment of the erythrocytes with H_2O_2 caused formation of methaemoglobin. Haemolysis was not observed.

Rat erythrocytes

Mg^{2+} efflux

As shown in figure 1, Mg^{2+} efflux in NaCl medium is strongly reduced by H_2O_2. After addition of 20 µmol/l H_2O_2, Mg^{2+} efflux in the absence and presence of amiloride was almost identical, indicating that amiloride-sensitive Mg^{2+} efflux was almost completely abolished.

At the same time, Na^+-independent Mg^{2+} efflux in sucrose medium and in choline chloride medium was increased by H_2O_2; at 20 µmol/l H_2O_2 the value for Mg^{2+} efflux was the same as that in NaCl medium (fig. 2, 3). These results indicate that after addition of 20 µmol/l H_2O_2, Mg^{2+} efflux was entirely Na^+-independent. Hence, Na^+/Mg^{2+} antiport was completely eliminated by H_2O_2.

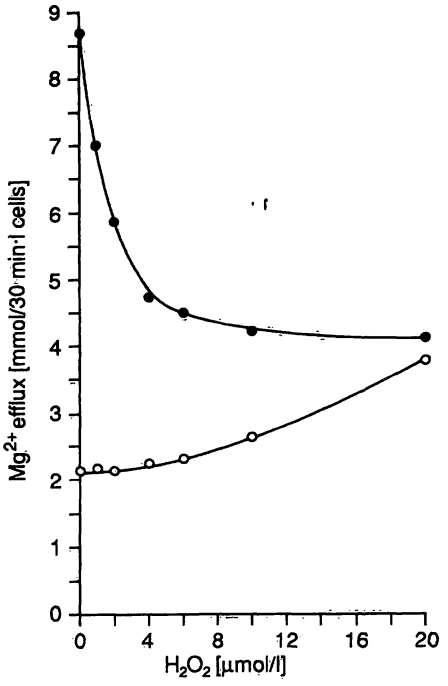

Fig. 1 Effect of H_2O_2 on Mg^{2+} efflux from Mg^{2+}-loaded rat erythrocytes in NaCl medium without amiloride (●) and in the presence of 1 mmol/l amiloride (○). Mean of 2 experiments. Na^+/Mg^{2+} antiport is given by the difference of Mg^{2+} efflux in NaCl=choline chloride medium (fig. 3).

In parallel with the increase of Na^+-independent Mg^{2+} efflux, the inhibition of this transport system by 4-acetamino-4'-isothiocyanato stilbene-2,2'-disulphonate (SITS) disappeared after addition of 20 µmol/l H_2O_2 (figs. 2, 3).

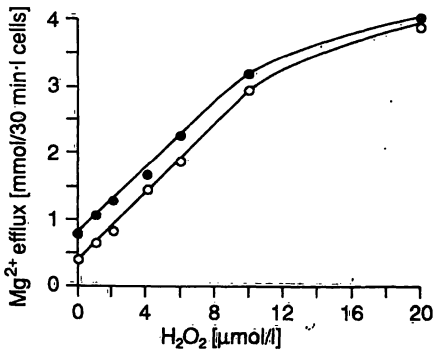

Fig. 2 Effect of H_2O_2 on Mg^{2+} efflux from Mg^{2+}-loaded rat erythrocytes in sucrose medium without (●) and in the presence of 30 µmol/l 4-acetamino-4'-isothiocyanatostilbene-2,2'-disulphonate (○). Mean of 2 experiments.

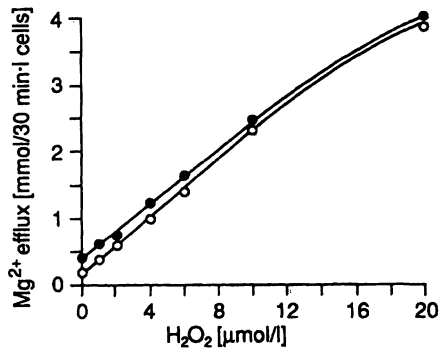

Fig. 3 Effect of H_2O_2 on Mg^{2+} efflux from Mg^{2+}-loaded rat erythrocytes in choline chloride medium without (●) and in the presence of 30 µmol/l 4-acetamino-4'-isothiocyanatostilbene-2,2'-disulphonate (○). Mean of 2 experiments.

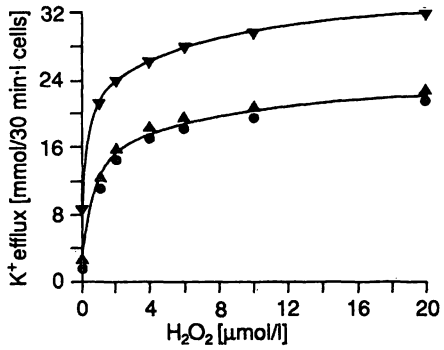

Fig. 4 Effect of H_2O_2 on K^+ efflux from Mg^{2+}-loaded rat erythrocytes. The cells were incubated in sucrose (▼), NaCl (●) and choline chloride (▲) medium. Same experiments as in figs. 1–3.

K^+ efflux

The effect of H_2O_2 on Mg^{2+} efflux was not specific. K^+ efflux, which is higher in sucrose than Cl^--containing media (4), was also drastically increased by H_2O_2 (fig. 4). K^+ efflux was more sensitive than Mg^{2+} efflux to the effect of H_2O_2.

The unspecificity of leak efflux was additionally shown by the increase of substances in the incubation medium that absorb at 260 nm (data not shown).

Human erythrocytes

Mg^{2+} efflux

The low rate of Mg^{2+} efflux from human erythrocytes is less accurately measurable than the faster Mg^{2+} efflux from rat erythrocytes.

The effect of H_2O_2 on Mg^{2+} efflux in human erythrocytes is in agreement with its effect on rat erythrocytes.

The low rate of Na^+-independent Mg^{2+} efflux in choline medium, which is inhibited by SITS was increased by H_2O_2 and no longer inhibited by SITS.

The low rate of Na^+-dependent amiloride-sensitive Mg^{2+} efflux (Na^+/Mg^{2+} antiport) disappeared after H_2O_2 treatment. Mg^{2+} efflux in sucrose medium was less inhibited by H_2O_2 (tab. 1).

K^+ efflux

As shown in table 2, H_2O_2 also increased K^+ efflux from human erythrocytes in NaCl and choline chloride media. The high rate of K^+ efflux from human erythrocytes in sucrose medium was inhibited to a small extent by H_2O_2.

Tab. 1 Effect of H_2O_2 (6 µmol/l) on Mg^{2+} efflux from Mg^{2+}-loaded human erythrocytes. Values in mmol/ 30 min × 1 cells. Mean ± SEM of 4 experiments.
Significant differences from controls by unpaired *Student*'s t-test. b, $p < 0.01$; c, $p < 0.001$.

Medium	Inhibitor	Control	H_2O_2
NaCl*	–	0.20 ± 0.01	0.29 ± 0.02[b]
NaCl	Amiloride (1.0 mmol/l)	0.10 ± 0.01	0.28 ± 0.02[c]
Sucrose	–	0.52 ± 0.03	0.37 ± 0.01[b]
Sucrose	SITS** (30 µmol/l)	0.10 ± 0.01	0.29 ± 0.01[c]
Choline chloride	–	0.09 ± 0.01	0.29 ± 0.01[c]
Choline chloride	SITS** (30 µmol/l)	0.06 ± 0.01	0.28 ± 0.02[c]

* The rate of Na^+/Mg^{2+} antiport is given by the difference of Mg^{2+} efflux in NaCl minus choline chloride medium.
** 4-acetamino-4'-isothiocyanatostilbene-2,2'-disulphonate.

Tab. 2 Effect of H_2O_2 (6 µmol/l) on K^+ efflux from Mg^{2+}-loaded human erythrocytes. Same experiments as in table 1. Values in mmol/ 30 min × 1 cells. Mean ± SEM of 4 experiments.
Significant differences from controls by unpaired *Student*'s t-test. c, $p < 0.001$.

Medium	Inhibitor	Control	H_2O_2
NaCl*	–	1.1 ± 0.1	4.9 ± 0.2[c]
NaCl	Amiloride (1 mmol/l)	1.0 ± 0.1	4.7 ± 0.3[c]
Sucrose	–	19.8 ± 0.7	14.2 ± 0.4[c]
Sucrose	SITS** (30 µmol/l)	4.2 ± 0.2	8.0 ± 0.4[c]
Choline chloride	–	1.1 ± 0.1	4.9 ± 0.5[c]
Choline chloride	SITS** (30 µmol/l)	0.6 ± 0.1	4.8 ± 0.4[c]

* The rate of Na^+/Mg^{2+} antiport is given by the difference of Mg^{2+} efflux in NaCl minus choline chloride medium.
** 4-acetamino-4'-isothiocyanatostilbene-2,2'-disulphonate.

Discussion

The effects of H_2O_2 on Na^+/Mg^{2+} antiport and Na^+-independent Cl^--accompanied Mg^{2+} efflux were completely different from the alteration of both Mg^{2+} efflux systems in erythrocytes from patients with cystic fibrosis. Therefore, the increase in Na^+/Mg^{2+} antiport in cystic fibrosis patients with score 3 is not caused by oxygen radicals, although in cystic fibrosis patients the formation of free radicals is increased (10). Hence, increased formation of free radicals is not involved in the increase of Na^+/Mg^{2+} antiport in erythrocytes from cystic fibrosis patients. Probably, the rate of oxygen radical formation in cystic fibrosis patients is not sufficient to injure erythrocyte membranes. The mechanism, by which Na^+/Mg^{2+} antiport is increased in cystic fibrosis patients, is still unknown.

In cultured amnion cells, oxygen radicals, formed by addition of xanthine oxidase plus xanthine, reduced the concentration of intracellular free Mg^{2+} (13). Our results indicate that this effect is due to Na^+-independent Mg^{2+} efflux.

Either an existing Cl^--associated Mg^{2+} efflux was enhanced or, more probably, another Mg^{2+} efflux was induced by the action of oxygen radicals. The latter suggestion agrees with the result that the newly-induced Mg^{2+} efflux was not inhibited by SITS.

In analogous experiments with human erythrocytes treated with *t*-butylhydroperoxide and azide, membrane holes with a radius of 0.5 nm were formed (16). The formation of these leaks was not correlated with oxidative denaturation of haemoglobin and oxidation of membrane protein SH-groups (16). However, leak formation was correlated to lipid peroxidation as monitored by the occurrence of malondialdehyde (16).

These membrane holes were permeable to small anions such as Cl^-, alkali cations, and to somewhat larger non-electrolytes (16).

The much higher K^+ than Mg^{2+} efflux induced by H_2O_2 is in agreement with this mechanism of H_2O_2-induced membrane holes, which discriminate between different diffusing sodium halides (16). Formation of membrane holes by H_2O_2 is also indicated by unspecific leakage of substances absorbing at 260 nm.

The smaller effect of H_2O_2 in sucrose medium may be due to radical scavenging by sucrose.

In cultured renal and cardiac cells, H_2O_2 induced membrane depolarization (17), which may favour Na^+-independent Mg^{2+} efflux.

As an alternative mechanism to the formation of membrane holes, it was found that *t*-butylhydroperoxide may activate K^+ channels in liver cells (18). Whether these K^+ channels are permeable to Mg^{2+} was not tested (18).

The reduction of Na^+/Mg^{2+} antiport and the reduction of SITS inhibition of Na^+-independent Mg^{2+} efflux by H_2O_2 suggest inactivation of the corresponding membrane proteins by H_2O_2 or oxygen radicals.

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